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OIT3 deficiency impairs uric acid reabsorption in renal tubule

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ABSTRACT

The oncoprotein induced transcript 3 (OIT3), also named liver-specific zona pellucida domain-containing protein (LZP), has been shown to be expressed in kidney, and was confirmed to interact with the Tamm–Horsfall glycoprotein (THP). However, the function of OIT3 in kidney remains unclear. In this study we found that serum uric acid level of *Oit3* null mice was significantly lower than that in wild type controls, whereas the excretion of uric acid in urine increased in the mutant mouse. Significantly, the excretion of THP in urine also increased while renal THP decreased in *Oit3* null mice. Our data suggest that OIT3 could maintain urate homeostasis by regulating the excretion and reabsorption of uric acid in renal tubule via cooperating with THP.

1. Introduction

Mammalian proteins with zona pellucida (ZP) domain are designated as ZP domain-containing proteins. These proteins are characterized by an extracellular motif of 260 amino acid residues, the ZP domain, which is thought to act as a polymerization module, promoting the formation of homo- or heterotypic filaments [1]. The first ZP protein, ZP1 was cloned from oocytes and shown to be involved in the acrosome reaction [2]. Since then, more and more ZP domain-containing proteins have been cloned from multiple tissues or cells. Recently, we identified the human and mouse liver-specific zona pellucida domain-containing protein (LZP), also named oncoprotein induced transcript 3 (OIT3), which functions as a secreted protein [3,4]. In our extending study, this gene was found to be expressed in mouse and rat kidney, where OIT3 was co-localized with Tamm–Horsfall protein (THP) in the renal thick ascending limb of Henle's loop (TAL). The in vivo interaction between OIT3 and THP was further confirmed in kidney and urine by co-immunoprecipitation assay, and the in vitro interaction was detected by GST pull-down assay [5].

THP, also called uromodulin, contains a complex glycosyl moiety with variable structure and is expressed in distal convoluted tubule and the thick ascending limb of renal tubules, as well as excreted in urine [6]. THP null mice showed inability to concentrate urine and a tendency for calcium oxalate stone formation, which suggested its role during normal renal physiology [7,8]. Moreover, the ability to clear urinary tract bacteria was also impaired in the THP null mice [9]. Given the interaction between OIT3 and THP proteins in kidney, we suspected OIT3 might be involved in normal kidney physiology and also wanted to know whether the putative function of OIT3 in kidney works through a THP interaction.

2. Materials and methods

2.1. Generation of Oit3 null mice

The *Oit3* null mice were generated by collaborating with the Shanghai Research Center for Model Organisms. The mouse *Oit3* gene was isolated from BAC clones (Geneservice, bMQ-207B8). A replacement targeting vector pBR322-MC1-hsvTK was constructed incorporating a 7.2 kb 5′ arm and 3.4 kb 3′ arm flanking a neomycin resistance cassette replacing the first two exons of the *Oit3* gene (Fig. 1A). The linearized vector was electroporated into embryonic stem cells (PluriStem 129/S6 Murine ES cells, Chemicon, Billerica, MA, USA), obtained from mouse strain 129/S6/SvEv. Successfully transfected embryonic stem cells were injected into developing blastocysts from C57Bl/6 J mice to obtain chimeric mice. Chimeric mice were bred with C57Bl/6 J female mice and screened by polymerase chain reaction (PCR) of tail DNA to obtain mice heterozygous for OIT3 deficiency. All animal experiments described herein were approved by the Shanghai Institutional Animal Care and Use Committees (protocol numbers 2010-0006). Mice were

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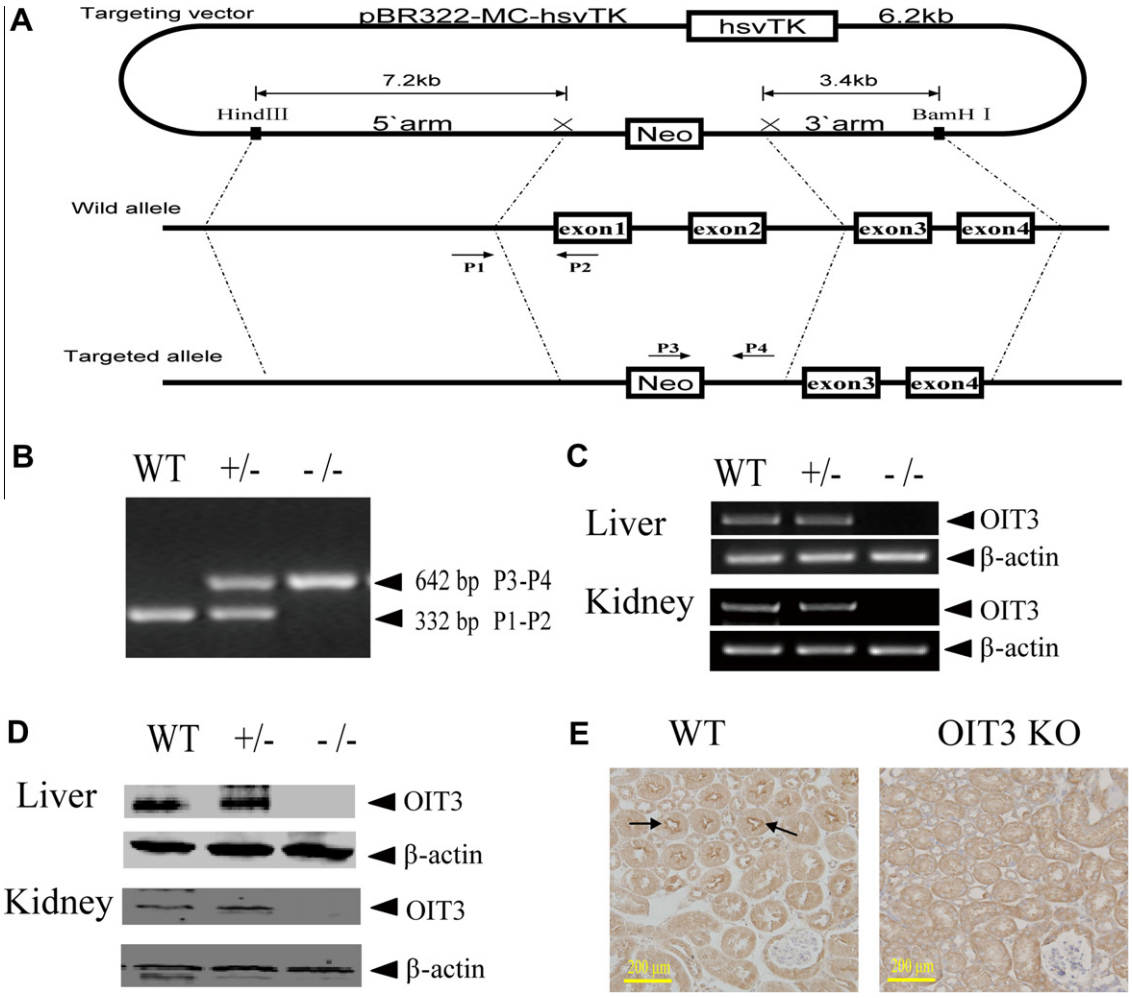

Fig. 1. Establishment of the *Oit3* null mouse model. (A) Strategy for disrupting the mouse *Oit3* gene. The *Oit3* targeting construct was 13.9 kb, consisting of a 7.2 kb 5′-arm, a 3.4 kb 3′-arm, and a 3.3 kb neomycin resistance cassette (Neo) replacing exons 1 and 2. The targeting vector also contained a further 6.2 kb including the Herpes Simplex virus thymidine kinase gene (TK). (B) The DNA from tail biopsies was amplified by PCR for identification. *Oit3* null mice have a product of 642 bp, while wild type mice have a band corresponding to 333 bp. Heterozygous mice have both bands. (C and D) mRNA and protein expression of OIT3 in liver and kidney were analyzed by RT-PCR and Western blotting assays, respectively. (E) Immunohistochemical staining on kidney from wild type and *Oit3* null mice. OIT3 locates within renal tubules in WT mice, as indicated by arrows.

group-housed under a 12-h light/dark cycle with unrestricted access to food and water. The heterozygous mice were bred with each other to obtain $Oit3^{+/+}$ and $Oit3^{-/-}$ offspring. The absence of OIT3 was confirmed by PCR, Western blot, and by immunohistochemistry. The *Oit3* null mice revealed no gross anomalies. They grew and bred normally.

2.2. Extraction of DNA, RNA and RT-PCR

The mouse DNA for PCR genotyping was from the tail and extracted with Qiagen DNA Extraction Kit. Total RNA was extracted with TRizol reagent (Invitrogen) based on manufacturer's protocol. RT reactions were carried out at 37 °C for 1 h in 20 μl reaction mixtures containing 1 μg total RNA, 10 pmol random primers, 200 units SuperScript II RT (Promega). The primers used in PCR were as followed: WT allele Forward: AAGGCTCCGTTACAGGCAGTT and Reverse: CA GCTGCCAGGAGTGGATAG or the neomycin resistance gene Forward CTGAGCCCAGAAAGCGAAGGA and Reverse: CACCCA TTGGTCA CTACAC. The primers for OIT3 mRNA identification Forward: CTGAAGATAATCACACTTGCCAAGTC and Reverse: CGATGTT GCTGGCCA CGATTTT. The PCR products were separated by electrophoresis on a 2% agarose gel. Samples were also analyzed with SYBR green-based qPCR performed on a TAKARA Sequence Detection System. The primers used in qPCR were as followed: THP Forward: TCAGCCTGAAGACCTCCC TA, Reverse: GAAAAGCCT-CAGTGGACAGC; Xanthine Oxidase Forward: CCACCCTGCTCTTTCA-GAAG, Reverse: TTCTCATAGCGGGG AATGTC; Urate Oxidase Forward: GGCCCTATGACAAAGGTGAA, Reverse: GCAGCAAAACCTCTT-CCTTG. All values were normalized to β-actin levels as previously described [10]. The mRNA level of gene expression was calculated as $2^{-\Delta\Delta CT}$.

2.3. Western blotting (WB) assay

Protein samples were separated by 8% SDS-PAGE, and then transferred to Hybond-C extra nitrocellulose membrane (Amersham Pharmacia Biotech). After being blocked in PBS containing 5% non-fat milk powder and 0.5% Tween-20, the membrane was incubated with rabbit anti-OIT3 antibody [3], mouse anti-THP antibody (Santa Cruz), rabbit anti-URAT1 (Abnova) and mouse anti-β-actin (Santa Cruz) at room temperature for 2 h, followed after washing by incubation with IRDye^{TM}800-labeled secondary antibodies (Li-Cor Biosciences Inc., Lincoln, Nebraska) for 1 h at room temperature. The signals were detected by Odyssey infrared imaging

system (Li-Cor Biosciences Inc.). The quantitative ratios are shown as relative optical densities of bands normalized to the expression of β-actin (kidney). The density of urine THP is estimated based on the Western blotting on the same amount of urine.

2.4. Immunohistochemistry assays

For immunohistochemistry analysis, 5-μm-thick paraffin-embedded sections were deparaffinized and pretreated with methanol containing 0.3% H_2O_2 to quench endogenous peroxidase activity. After incubating in 0.01 M Sodium Citrate Solution buffer (pH 6.0) at 85 °C for 15 min, the sections were blocked with 20% goat serum in PBS at 37 °C for 1 h. Then the sections were incubated with anti-OIT3 polyclonal antibodies at 4 °C overnight, followed by incubation with the horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies (DACO, Kyoto, Japan) at 37 °C for 1 h. The signals were detected with Diaminobenzidine (DAB) Substrate Kit (Vector Laboratories, Inc., Burlingame, CA) according to manufacturer's instructions.

2.5. Analyses of serum and urine

The mice were used at age 6–8 weeks. The animals received standard mouse chow and were allowed drinking water ad libitum. Each group had 6–8 mice and mice were placed individually into metabolism cages. Urine volume, osmolality (freezing point depression), sodium, chloride, potassium, creatinine, uric acid, and total protein were determined by routine automated methods. The urine samples were checked for the presence of THP by Western Blot. Thereafter, animals were killed after anesthesia with 4% chloral hydrate intraperitoneal injection (0.01 ml/g). Blood was taken from mouse orbit and centrifuged at 1000×*g* at room temperature. Serum was collected and stored in −80 °C. The sera were analyzed with an autobiochemistry instrument (Hitachi 7600, Japan).

2.6. Statistical analysis

Data are shown as means ± S.D. and were analyzed by using an ANOVA test and Mann–Whitney U test.

3. Results

3.1. Establishment of an Oit3 null mouse model

We generated *Oit3* null mice by targeting the first two exons of the *Oit3* gene via homologous DNA recombination in embryonic stem (ES) cells (Fig. 1A). The mice were bred and genotyped by polymerase chain reaction (PCR). Genotyping primers were designed as shown in Fig. 1A. *Oit3* wild type (WT) mouse were identified by the presence of one single DNA product of 333 bp, whereas heterozygous (+/−) and homozygous (−/−) mice had a product of 642 bp (Fig. 1B). As shown in Fig. 1C, OIT3 mRNA expression was absent in liver and kidney of homozygous knockout mice (−/−) as determined by RT-PCR. The absence of OIT3 protein expression in the mutant mice was confirmed by Western blotting (Fig. 1D). Moreover, anti-OIT3 antibody immunohistochemistry staining also showed that the OIT3 signal was localized in the luminal membrane of renal tubule in WT mice but not in *Oit3* null mice (Fig. 1E). These results collectively indicated that *Oit3* null mice were successfully established in this study.

3.2. Serum uric acid level is reduced in Oit3 null mice

Within our observation periods, these *Oit3* null mice were viable and fertile without gross abnormalities. We also did not find any abnormal morphological manifestations. To explore the potential metabolic phenotypes in *Oit3* null mice, we assessed the activity of hepatic enzymes, AST and ALT, as well as the concentration of Na^+, K^+, and Cl^- in the serum of *Oit3* null mice. However, we found no significant difference between *Oit3* null mice and wild type littermates (Table 1).

Given that OIT3 is expressed in kidney, we next assessed the putative phenotypes involving kidney functions in *Oit3* null mice. The blood concentrations of creatinine (CRE), and urea nitrogen (BUN) were measured, and as with the previously mentioned Na^+, K^+, and Cl^- ions, exhibited no significant difference between *Oit3* null mice and wild type controls (Table 1). However, regardless of gender, the serum uric acid level was significantly reduced in *Oit3* null mice as compared to that in WT littermates ($P < 0.05$) (Table 1).

3.3. Excretion of water and uric acid is increased in Oit3 null mice

To explore the reason for the deceased serum uric acid level in *Oit3* null mice, we first evaluated the expression of xanthine oxidase (XO) and urate oxidase (UOX), which are known to be responsible for uric acid metabolism in mouse. We did not identify abnormal expression of these enzymes in *Oit3* null mice by RT-PCR assay (Fig. S1).

Next we checked the uric acid concentration in urine, because the renal tubule also regulates it via excretion and reabsorption. Interestingly, a significant increase of urine uric acid was found in female *Oit3* null mice (Table 2). Furthermore, total volume of urine excreted during a 24 h period, including the urine volume normalized by weight, was also significantly increased in female

Table 1
Serum indices related to liver and kidney functions in wild type and OIT3 null mice.

Items	Male		Female	
	Wild type	Homozygous	Wild type	Homozygous
Albumin (g/l)	33 ± 1.67	29.67 ± 7.03	35 ± 2.45	34.5 ± 2.08
Globulin (g/l)	20 ± 3.09	21 ± 7.84	15.5 ± 2.08	18.25 ± 3.86
AST (u)	91.83 ± 21.57	98.7 ± 26.52	91 ± 23.15	99.7 ± 13.15
ALT (u)	43.68 ± 20.72	40.2 ± 13.07	41.6 ± 20.14	36.85 ± 8.26
Cholesterol (mmol/l)	3.45 ± 0.84	3.34 ± 0.4	3.11 ± 0.44	3.39 ± 0.63
Na^+ (mmol/l)	147.25 ± 2.68	146.8 ± 2.04	148 ± 2.94	144 ± 4.58
K^+ (mmol/l)	4.76 ± 0.17	4.69 ± 0.57	4.56 ± 0.39	4.84 ± 0.34
Cl^- (mmol/l)	108.25 ± 2.04	104.8 ± 3.56	105.75 ± 2.5	106 ± 4.58
Uric acid (μmol/l)	127.58 ± 52.04	96.86 ± 25.22*	96.0 ± 22.7	71.89 ± 16.43**
Creatinine (μmol/l)	15.64 ± 5.17	14.9 ± 4.44	12 ± 1.95	13.04 ± 4.63
BUN (μmol/l)	10.14 ± 2.8	10.33 ± 1.57	10.02 ± 1.87	9.76 ± 3.37

Values are means ± S.D.; n = 8–10/genotype and gender. Statistical analysis was performed using ANOVA test. * and ** indicated $P < 0.05$ and $P < 0.01$ for KO mice vs wild type mice, respectively. AST, aspartate amino-transferase; ALT, alanine amino-transferase; BUN, blood urea nitrogen.

Table 2
Urinary indices related to kidney function in wild type and OIT3 null mice.

Items	Male		Female	
	Wild type	Homozygous	Wild type	Homozygous
Body weight (g)	23.02 ± 3.28	23.47 ± 1.00	18.6 ± 2.17	19.28 ± 0.66
Volume (ml/day)	0.64 ± 0.34	0.76 ± 0.34	0.515 ± 0.18	0.90 ± 0.43*
Volume (ml/g)	0.027 ± 0.015	0.032 ± 0.014	0.028 ± 0.011	0.044 ± 0.022*
Na^+ (μmmol/day)	186.92 ± 29.91	224.46 ± 112.64	124.7 ± 89.36	155.3 ± 21.9
K^+ (μmmol/day)	165.37 ± 34.28	216.28 ± 75.61	147.24 ± 83.68	205.28 ± 25.87
Cl^- (μmmol/day)	117.91 ± 26.76	140 ± 69.34	81.93 ± 46.8	100.56 ± 13.13
Creatinine (μmol/day)	3.77 ± 0.69	4.53 ± 0.93	3.26 ± 0.91	4.08 ± 0.93
Uric acid (nmol/g·day)	16.86 ± 8.52	19.32 ± 9.09	20.71 ± 7.76	32.73 ± 9.07**
Osmolality (mosmol/kgH_2O)	2325 ± 682.2	2333.3 ± 677.2	2206.7 ± 1284.4	2322.5 ± 767.6
Deprivation of water for 24 h				
Volume (ml/day)	0.31 ± 0.18	0.35 ± 0.23	0.25 ± 0.2	0.29 ± 0.11
Osmolality (mosmol/kgH_2O)	2688.3 ± 601.9	3240 ± 712.1	2527.5 ± 766.9	3896.7 ± 731.5

Values are means ± S.D.; n = 8–10/genotype and gender. Statistical analysis was performed using ANOVA test. * and ** indicated $P < 0.05$ and $P < 0.01$ for OIT3 null mice vs wild type mice, respectively.

Oit3 null mice as compared to that in WT mice. However, the ion concentrations of Na^+, K^+, and Cl^-, as well as urine osmolarity, had no significant change. Moreover, we also performed a water deprivation experiment to assess the water reabsorption of kidney. After 24 h water deprivation, average urine volume and osmotic pressure exhibited somewhat of an increase, but this was not statistically significant (Table 2).

3.4. Excretion of renal THP is enhanced in Oit3 null mice

As reported before, uric acid is increased in serum but decreased in urine in *Thp* mutant mice [11]. To address whether OIT3 functions through THP protein, we first assessed the mRNA level of THP, and found that THP transcription was not obviously changed in *Oit3* null mice (Fig. S2). Significantly, the THP protein level in kidney was decreased in *Oit3* null mice, while urine THP was increased, as shown by Western blotting assay (Fig. 2A, C D). Moreover, we also assessed the expression of URAT1 in *Oit3* null mice. URAT1 functions as a main urate/anion exchanger in the luminal membrane of the epithelium of renal proximal tubules, and plays a key role in transporting uric acid into cells from urine [12]. However URAT1 expression was not significantly altered in *Oit3* null mice, as compared to that of WT mice (Fig. 2A and B).

4. Discussion

In a quest to reveal the physiological function of OIT3, we successfully established the *Oit3* null mouse model in this study. Significantly, we propose that OIT3 could be involved in renal uric acid excretion due to the dysregulation of uric acid in serum and urine in the *Oit3* null mice.

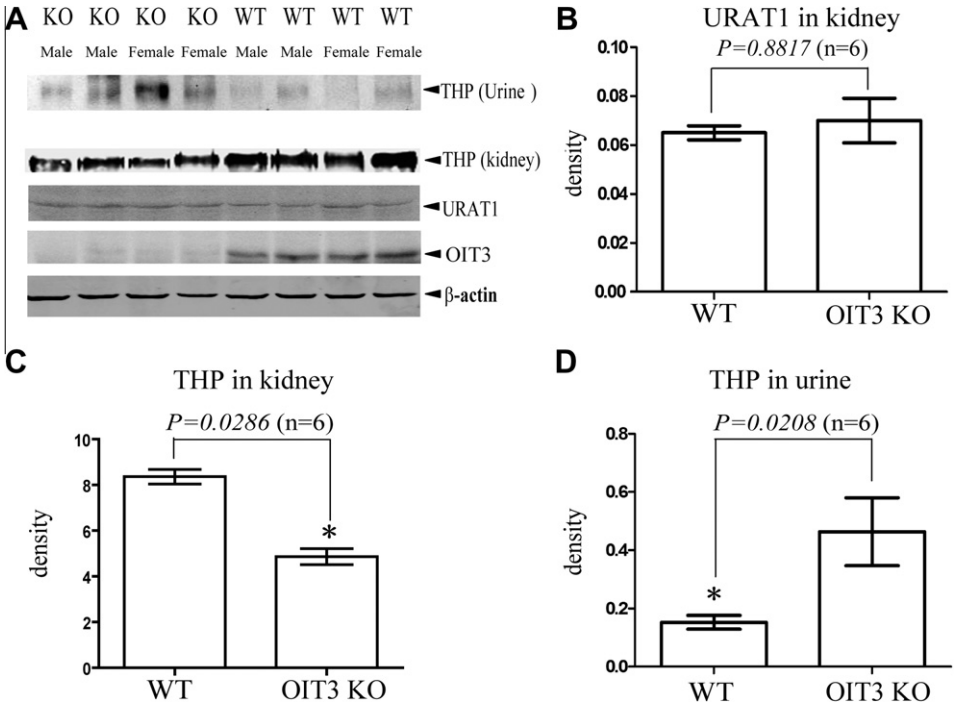

Fig. 2. THP levels in kidney and urine of OIT3 mull mice. (A) THP and URAT1 levels in kidney and urine were evaluated by Western blotting assay. Blots were quantified to compare levels of URAT1 (B) and THP (C) in kidney from wild type and *Oit3* null mice. (D) The level of THP in urine from wild type and *Oit3* null mice was also analyzed. * indicates $P < 0.05$, Mann–Whitney U test.

A similar phenotype was also seen in *Thp* null mice [8], where THP has been shown to be a partner of OIT3 in our previous work. Moreover, mutations in the THP gene were found in familial juvenile hyperuricaemic nephropathy (FJHN) [13,14], which revealed the central role of THP during uric acid metabolism [15]. Therefore, we suspected the role of OIT3 in renal tubule might work through the THP protein. Indeed, THP protein was decreased in kidney but increased in urine from *Oit3* null mice. Both OIT3 and THP belong to the ZP-containing protein family, a protein domain which might play a role in protein polymerization into filaments and/or matrics [16]. THP was observed to form filaments and could not be degraded by proteolytic digestion [17]. Herein, we assumed that OIT3 could bind to THP to form protein polymers, and hence stabilize THP within renal tubules, thus maintaining the urate homeostasis. However, as OIT3 was absent in the genetically engineered mice, the protein polymerization involving both OIT3 and THP would be disrupted, and thereby THP would be excreted to the urine, which could impair uric acid reabsorption by renal tubules. It should be pointed out that this hypothesis needs to be further validated via other animal models, including *Oit3* and *Thp* double knockout ($Oit3^{-/-}$ $Thp^{-/-}$) mice.

Multiple ion transporters are localized to the luminal surface of the thick ascending limb of Henle's loop [18], including NKCC2, ROMK, Na^+/H^+ exchanger (NHE3), KCC4 and CIC-Kb [19]. Even though it was reported before that the THP protein is co-localized with the NKCC2 [20] and potassium channel ROMK2 in renal outer medulla [21], we did not see a difference in serum or urinary Na^+, K^+, and Cl^- ions in *Oit3* null mice. We postulate that Na^+, K^+, and Cl^- ions are reabsorbed in other fractions of kidney, such as distal tubule and collecting tubule, not overlapping with the OIT3 distribution.

In this study, the enzymes involving uric acid synthesis were not significantly changed in *Oit3* null mice, suggesting that OIT3 would not regulate uric acid level through modifying uric acid synthesis. Here a significant increase of urine uric acid was found only in female *Oit3* null mice, which could be associated with androgen action on THP [22]. However, the molecular mechanisms by which sex hormones, androgen and estrogen, regulate renal uric acid excretion and reabsorption via OIT3 and THP are unclear, and needs to be further studied.

Nevertheless, our study still indicates a potential role of OIT3 in some clinical diseases involving uric acid metabolism. In humans, the main disease associated with uric acid dys-absorption is gout, whose symptom is hyperuricacidemia [23–25]. The treatment of gout is by consistently reducing the level of uric acid. Current urate-lowering drugs use two traditional strategies: inhibition of xanthine oxidase to reduce production of uric acid [26], using drugs such as allopurinol and febuxostat [27–29], and promotion of uricosuria to increase its renal excretion, with substances such as probenecid and benzbromarone [30]. Since the loss of OIT3 reduces uric acid level in serum and enhances the excretion of this metabolite, OIT3/LZP could be considered as a new therapeutic target for hyperuricacidemia.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2012.01.038.

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